Exercise intensity and duration effects on in vivo immunity.

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Exercise intensity and duration effects on in vivo immunity

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MSSE-D-14-00903, Responses to reviewers

Reviewer 1

Thanks for your helpful comments. We do hope you agree that the changes deal with your comments and improve the paper.

1. J shaped model: Good point. We have added text to the introduction accordingly (L24-27). We have also added, to paragraph 1 and 2 in the discussion, text stating that results from our 30MI trial do not indicate immune enhancement (paragraph 1) and thus do not support one important hypothesis underpinning the J curve (paragraph 2). Nieman reference added.

2. Rationale for choice of exercise intensities/durations: Thanks. We have added justification to paragraph 2 in the methods (L87-92).

3. Greenhouse-Geisser adjustment: Thanks. We have amended the statistical analysis section so it's clear where correction was applied (L219, statistical analysis section).

4 and 6: Agreement: Thanks for your comment. You are correct that we performed this analysis to assess the validity of using skin-fold calipers as a practical, albeit more subjective, method to determine dermal thickness. As you have suggested, we have now added this wording to the methods section (paragraph 2, statistical analysis section). However, please note, that these two measures are not directly comparable, since the skin-fold measure assesses a double thickness of skin, compared to the ultrasound measurement that assess a single thickness. It would therefore be inappropriate to directly compare these measures and calculate a bias (difference) between the two, and use the term "agreement". On recommendations of Hopkins et al 2009 (*MSSE*, 41, 3-12), who disapproves of the use of limits of agreement, we have presented the r and r squared value, along with the standard error of the estimate, which detail the degree of error in the prediction, as opposed to bias +/- limits of agreement. This information has been added to the methods (L240-245, statistical analysis section) and the results (L264-266). Thanks.

5. Fig 3: Thanks. We have changed Fig 3 so it now includes the pre-exercise values and conducted the statistics taking account of the extra time point. The results text has changed accordingly (See circulating stress hormones section).

Many thanks for your constructive comments. We do hope you agree that the changes deal with your comments and improve the paper.

Reviewer 2

Thanks for your helpful comments. We do hope you agree that the changes deal with your comments and improve the paper.

1. Introduction: Lines 64-66. Please develop further the argument why local cutaneous inflammatory responses might be hypothesized to be involved in the mechanism through which CHS is affected by exercise.

Thanks. Please see text in lines 49-61 describing the rationale for a possible involvement of local cutaneous inflammatory responses in the inhibition of CHS by prolonged exercise. In response to your suggestion, we have also added text (L52-55), to elaborate further.

2. No mention of females: Thanks for raising this. We have added this limitation to the discussion (L394-396).

3. Weekly exercise has now been added to Table 1. Thanks.

4. The time period between preliminary testing and experimental trial has now been added (L121).

5. Timing of sensitization and stress hormone kinetics: Thanks, this is a good point. We have added text to the discussion highlighting this limitation and have provided a rationale for our choice of sensitization timing 20 minutes after exercise (L368-382).

6. DPCP dose and response curves: Thanks. These have now been added (Fig. 2B) and the results text changed accordingly (results paragraph 1).

7. Brief psychological stress (15 min) has been found to reduce the DTH response (Altemus et al. 2006), this should be brought into the discussion of the extant literature.

Thanks. It's important to make the distinction (and to avoid confusion) between assessing the effects of stress on induction of (new) immunity as assessed here with DPCP and the effects of stress on the (secondary/tertiary) response to common recall antigens as performed by Altemus and colleagues. Altemus assessed the influence of 15 minutes psychological stress (trier social stress test) on DTH using common recall antigens (e.g. candida, tetanus toxoid and tricophyton). Hence, where possible, we have restricted direct comparisons of our findings to studies investigating the influence of stress (e.g. exercise) on the primary immune response. Nevertheless, the excellent paper by Altemus is indeed cited (reference 3, L12 and L367) and we have highlighted this in red in the introduction and discussion. Thanks again.

8. Request to reduce length of CHS perspectives section: Given the broad readership of MSSE we feel it's important to include a section that deals with perspectives (strengths, weaknesses, context etc.). Nevertheless, in response to your suggestion, we have now reduced this section in length. Thanks again.

Many thanks for your constructive comments. We do hope you agree that the changes deal with your comments and improve the paper.

Exercise intensity and duration effects on *in vivo* immunity

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ABSTRACT

Purpose: To examine the effects of intensity and duration of exercise stress on induction of *in-vivo* immunity in humans using experimental contact hypersensitivity (CHS) with the novel antigen Diphenylcyclopropenone (DPCP). **Methods:** Sixty-four healthy males completed either 30 minutes running at 60% $\dot{V}O_{2peak}$ (30MI); 30 minutes running at 80% $\dot{V}O_{2peak}$ (30HI); 120 minutes running at 60% $\dot{V}O_{2peak}$ (120MI) or seated rest (CON). Twenty-minutes later subjects received a sensitizing dose of DPCP and four-weeks later the strength of immune reactivity was quantified by measuring the cutaneous responses to a low, dose-series challenge with DPCP on the upper inner-arm. Circulating epinephrine, norepinephrine and cortisol were measured pre, post and 1h post-exercise or CON. Next, to better understand whether the decrease in CHS response on 120MI was due to local inflammatory or T-cell mediated processes, in a cross-over design, eleven healthy males performed 120MI and CON and cutaneous responses to a dose-series of the irritant, croton oil (CO) were assessed on the upper inner-arm. **Results:** Immune induction by DPCP was impaired by 120MI (skin-fold-thickness -67% *vs* CON; $P<0.05$). However, immune induction was unaffected by 30MI and 30HI despite elevated circulating catecholamines (30HI *vs* pre: $P<0.01$) and greater circulating cortisol post 30HI (*vs* CON: $P<0.01$). There was no effect of 120MI on skin irritant responses to CO. **Conclusions:** Prolonged, moderate-intensity exercise, but not short-lasting high or short-lasting moderate-intensity exercise, decreases the induction of *in-vivo* immunity. No effect of prolonged, moderate-intensity exercise on the skin's response to irritant challenge points towards a suppression of cell-mediated immunity in the observed decrease in CHS. DPCP provides an attractive tool to assess the effect of exercise on *in-vivo* immunity.

Key words: stress; running; immune; contact hypersensitivity; diphencyprone; irritant

INTRODUCTION

The skin constitutes the body's largest immunological organ, providing the first line of defense against pathogenic and environmental assaults (8). Measures of *in vivo* immunity at the skin include delayed type hypersensitivity (DTH) responses to intradermal injection of antigens, or the less invasive contact hypersensitivity (CHS) responses to epicutaneous application of antigens. These *in vivo* measures are considered more informative than the commonly used *in vitro* measures where immune cells, typically from peripheral blood, are extracted from their normal environment and analyzed in artificial cultures (2). Isolated measures of immune function may react differently to a whole-body immune challenge because they lack the highly integrated neural and hormonal components within the specific tissue environment in which immune responses usually take place (1). Studies using *in vivo* cutaneous immune measures have shown impaired responses in individuals exposed to psychological stress (3), physical stress (17), during acute infectious illness e.g. Epstein-Barr virus (5) and in diabetes and psoriasis (4). Furthermore, *in vivo* cutaneous immune measures have been shown to predict mortality in critically ill HIV-infected patients (12) and in patients with surgical infections (31). There is a need to better understand *in vivo* cutaneous immune measures for investigators examining the influence of exercise stress on immunity in humans.

Physical exercise provides a well-controlled model to study the effects of stress on immune responses. Given the obvious ethical constraints of studying experimental infection in humans, animal models have provided valuable insight into the effects of exercise on clinically relevant responses to viral infection. The work in animals indicates that prolonged and high intensity exercise is associated with higher mortality rates whereas short, moderate intensity exercise lowers mortality rates, compared with controls (21). The research evidence on immune responses after short, moderate intensity exercise in humans is not definitive and tends to indicate immune enhancement only in individuals with sub-optimal immune status (14, 30).

Work in humans indicating that a single bout of short duration, high intensity exercise and prolonged duration, moderate intensity exercise decreases immunity, is largely based upon results of studies examining *in vitro* immune measures (26, 32). Little is known about the impact of a single bout of exercise on cutaneous measures of *in vivo* immunity in humans. One such study showed that after an acute bout of prolonged, continuous exercise (lasting ~6.5 h), DTH reactions to common recall antigens in the Mérieux CMI Multitest® were reduced but this test is no longer commercially available (6). Moreover, the use of common recall antigens does not permit the assessment of the effects of stress on the induction of new immune memory and findings may be confounded by the lack of control over immunological memory: both the sensitizing dose and time elapsed since sensitization influence immunological memory. To the best of our knowledge, no study has investigated the impact of the intensity and duration of continuous exercise stress on *in vivo* immunity in humans. Challenging the skin using novel antigens such as keyhole limpet hemocyanin (KLH) (35) or diphenylcyclopropenone (DPCP) (17) permits the investigation of the influence of stressors on *in vivo* immunity and allows rigorous control of both the dose and timing of sensitization. Using topical DPCP, we have recently shown that 2 h of moderate intensity exercise decreases both the induction of immunity (-53%) in those with no prior exposure to DPCP and elicitation of immunity (-19%) in those who received repeated monthly DPCP exposures to boost responses to a reproducible plateau (17). Possible mechanisms include the activation of the hypothalamic-pituitary-adrenal axis and sympatheticoadrenal-medullary axis, which is widely acknowledged to occur following prolonged stress (typically lasting hours) and in-turn increases glucocorticoids and catecholamines, previously shown to decrease the induction of CHS in mice (10, 33). It has yet to be determined whether the inhibitory effects of prolonged exercise on immune responses to DPCP are due to systemic effects on the dendritic cell/T cell axis between the skin and lymph nodes or whether they involve local effects on cutaneous inflammatory processes mediated principally via innate immune mechanisms. The levels of

local cutaneous cytokines known to facilitate (e.g. IL-1β) and inhibit (e.g. IL-10) dendritic cell (DC) migration are considered to play a central role in the early DC-dependent events of CHS induction, namely, antigen processing and DC trafficking (38). One experimental approach to this problem is to investigate the effect of prolonged, moderate intensity exercise on cutaneous responses to a topically applied irritant such as croton oil (CO). Unlike DPCP, which ultimately stimulates an antigen-specific, T-cell-mediated immune response, CO is an irritant, which stimulates a non-T-cell mediated, inflammatory response after a single exposure (27). CO has no sensitizing properties but is capable of producing similar cutaneous erythema responses to those seen after CHS challenge (40).

Here we present the findings from two studies, starting with the effects of intensity and duration of exercise stress on *in vivo* immune induction by DPCP. We hypothesized that a prolonged, moderate intensity exercise bout (120 minutes at 60% $\dot{V}O_{2peak}$) and a short, high intensity exercise bout (30 minutes at 80% $\dot{V}O_{2peak}$) would decrease the CHS responses to DPCP compared with a short, moderate intensity exercise bout (30 minutes at 60% $\dot{V}O_{2peak}$) and seated rest. Then, to examine whether exercise-related effects on local cutaneous inflammatory processes play a role in the inhibitory effect of prolonged, moderate intensity exercise on the CHS response we investigated irritant responses to a CO patch test.

METHODS

Subjects. All subjects were healthy, non-smoking, recreationally active males with no previous history of exposure to DPCP. Subjects were excluded if they were taking any medication or dietary supplements, had a history of atopy or any other immune-related or inflammatory dermatological conditions. Subjects were required to abstain from caffeine, alcohol, and exercise for 24 h before and 48 h after the experimental trials. All subjects gave written informed consent to participate after being fully briefed and informed of the study's procedures. The study received Local University Ethics Committee approval and was conducted in accordance with the Declaration of Helsinki principles.

The effect of exercise intensity and duration on induction of DPCP immune memory. Subjects were matched for age and aerobic fitness (gas exchange threshold (GET) and $\dot{V}O_{2peak}$) before being randomly assigned to one of four experimental groups: 1) 120 minutes of seated rest (CON); 2) 30 minutes of moderate intensity (60% $\dot{V}O_{2peak}$) exercise (30MI); 3) 30 minutes of high intensity (80% $\dot{V}O_{2peak}$) exercise (30HI); or 4) 120 minutes moderate intensity (60% $\dot{V}O_{2peak}$) exercise (120MI) (Fig. 1). These exercise intensities and durations were chosen to allow comparison with the relevant literature (17), to assess the *in vivo* immune response to exercise recommended to healthy adults for fitness and health (e.g. the ACSM recommends 30 minutes, moderate-intensity exercise on most days), to best separate intensity and duration effects on *in vivo* immunity; and finally, with feasibility in mind (e.g. our subjects could complete 30 minutes at 80% $\dot{V}O_{2peak}$). There were no significant differences between groups for characteristics (Table 1). The study was performed between February, 2011 and April, 2012 and no data was taken from our previous investigation that also included 120MI and CON trials (17).

*** Fig. 1 near here ***

*** Table 1 near here ***

Preliminary measures and familiarization. Anthropometric measures were recorded on arrival at the laboratory. Body composition assessment was completed by whole body Dual Energy X-ray Absorptiometry (DEXA: Hologic QDR Series-4500, USA). Following this, $\dot{V}O_{2peak}$ was estimated by means of a ramped exercise test on a treadmill (h/p/cosmos Mercury 4.0, Nussdorf-Traunstein, Germany). Following 3- minutes of walking at 5 $km \cdot h^{-1}$ with an incline of 1 %, speed increased at a rate of 1 $km \cdot h^{-1} \cdot min^{-1}$ to a maximum of 18 $km \cdot h^{-1}$, after which the incline increased at a rate of 1 $\% \cdot min^{-1}$ until volitional exhaustion. Pulmonary gas exchange was measured breath-by-breath for the duration of the test (Cortex Metalyser 3B, Biophysik, Leipzig, Germany). The $\dot{V}O_{2peak}$ was taken as the highest 30-s average value before the subject's volitional exhaustion and the speed equivalent to 60 % or 80 % of the $\dot{V}O_{2peak}$ was calculated. The GET was also determined from the ramped exercise test using the V-slope method.

At least 24 h after the preliminary test, each subject's calculated exercise intensity was verified by running for 50 % of their allocated exercise duration and all subjects were familiarized with laboratory equipment.

Experimental procedures. Dietary intake was controlled during the 24 h before the main experimental trial by providing subjects with their estimated daily energy requirement using DEXA determined fat free mass as described (mean ± SD: 11.2 ± 1.1 MJ day^{-1}) (9), multiplied by a physical activity factor (37), and water proportional to 35 $mL \cdot kg^{-1} \cdot d^{-1}$ body mass.

Within 3 weeks of the preliminary testing, on the day of the exercise trial, all subjects were transported to the laboratory at 0730 h and provided with a standard breakfast (0.03 $MJ \cdot kg^{-1}$). Subjects were permitted to perform light activity before commencing the intervention. Nude body mass (NBM) was recorded before and after exercise on a digital platform scale to determine water allowance (Model 705; Seca, Hamburg, Germany). Exercising subjects received 5$ml \cdot kg^{-1}$NBM of water immediately before and after the exercise, 2 $ml \cdot kg^{-1}$NBM at 15 minutes intervals throughout, and any additional exercise fluid loss was replaced following exercise. Subjects assigned to the 120MI began running on a treadmill at 1100 h and those assigned to 30HI and 30MI began at 1230 h, so that all subjects completed the exercise at the same time of day (1300 h; Fig. 1). Immediately after the trial, exercising subjects showered and returned to the laboratory within 15 minutes of completion. The CON, non-stress condition, consisted of 2 h seated, passive rest in the same laboratory, in the same ambient conditions of 20 °C, at the same time of day, with a fluid intake proportional to 35 $mL \cdot kg^{-1} \cdot d^{-1}$ body mass.

Induction of contact sensitivity. Subjects were sensitized to DPCP at 1320 h, exactly 20 minutes after exercise cessation or equivalent seated rest, as described previously (17). This sensitization time was chosen to allow cutaneous blood flow to return to baseline (19). The sensitizing exposure to the novel antigen DPCP involved application of an occluded patch, constituting a 12 mm aluminum Finn chamber (Epitest Oy, Tuusula, Finland) on scanpor hypoallergenic tape containing an 11 mm filter paper disc. The paper disc was soaked in 22.8 µl of 0.125 % DPCP in acetone (patch = 30 $\mu g \cdot cm^{-2}$ DPCP) and allowed to dry for 5 minutes before being applied to the skin on the lower back, for exactly 48h.

Elicitation. The magnitude of *in vivo* immune responsiveness was quantified by measuring the responses elicited by secondary exposure to the same antigen (Fig. 1). Twenty eight days after the initial sensitization to DPCP, all subjects received a challenge with a low concentration,

dose-series of DPCP on individual patches, each comprising an 8mm aluminium Finn chamber on scanpor hypoallergenic tape containing a 7 mm filter paper disc. Patches were applied to the inner aspect of the upper arm in the following concentrations: 10 µl of DPCP: 0.0048 %, 1.24 $\mu g \cdot cm^{-2}$; 0.0076 %, 1.98 $\mu g \cdot cm^{-2}$; 0.012 %, 3.17 $\mu g \cdot cm^{-2}$; 0.0195 %, 5.08 $\mu g \cdot cm^{-2}$; 0.0313 %, 8.12 $\mu g \cdot cm^{-2}$ and 10 µl 100 % acetone control patch for background subtraction. Patches were applied in randomly allocated order at the local site in order to minimize any anatomical variability in responses. Elicitation patches were removed after 6 h and the strength of immune reactivity was assessed as cutaneous responses at 48 h post-application.

Blood collection and analysis. Blood samples (venepuncture from an antecubital vein) were collected into one K_3EDTA coated vacutainer, and one lithium heparin coated vacutainer (Becton Dickinson, Oxford, UK) pre, immediately post and 1 h post exercise. The samples were spun at 1500 *g* for 10 minutes in a refrigerated centrifuge. Plasma was aliquoted into Eppendorf tubes, and immediately frozen at -80°C for later analysis.

Plasma epinephrine and norepinephrine concentrations in K_3EDTA plasma were determined using a commercially available CatCombi ELISA (IBL International, Hamburg, Germany). Aliquots of lithium heparin plasma were used to determine cortisol concentration by ELISA, performed according to the manufacturer's instructions (DRG Instruments, Marburg, Germany). The intra-assay coefficient of variation for plasma epinephrine, norepinephrine and cortisol was 4.1 %, 4.1 % and 4.4 %, respectively.

The effect of prolonged, moderate intensity exercise on the cutaneous response to the irritant, croton oil. To investigate the possibility that the inhibitory effect of 120MI on CHS induction was mediated via local effects on cutaneous inflammatory processes, 11 healthy

172 males (age 24 ± 5 years; height 179 ± 8 cm; body mass 79.0 ± 9.9 kg; $\dot{V}O_{2peak}$ 53 ± 6 ml.kg^{-1}.min^{-1}) completed a follow-up study to investigate the cutaneous responses to the non-specific
174 irritant, CO.

175

176 In a randomized, counterbalanced, repeated measures design, subjects performed 120MI-CO or
177 CON-CO separated by 7 - 14 d. Subjects received a CO challenge at 1320 h, exactly 20
178 minutes after exercise cessation or seated rest. This involved the topical application of a dose-
179 series of CO on individual patches comprising 8mm aluminium Finn chambers mounted on
180 hypoallergenic scanpor tape and 7 mm filter paper discs. Patches were applied in duplicate to
181 the inner aspect of the upper arm in the following concentrations: 10 µl of CO in ethanol: 0.3
182 %, 0.55 %, 1.0 % and 3 % and 10 µl 100 % ethanol control patch (23). To account for local
183 anatomical variability, the location of each concentration was randomized. Patches remained in
184 place for exactly 24 h and the assessment of cutaneous responses was performed 2 h after
185 removal of the CO patches, as described (23).

186

187 **Assessment of cutaneous responses.** Skin edema (inflammatory swelling) is considered the
188 key measure of CHS elicitation responses (17). This was assessed as mean skin-fold thickness
189 from triplicate measurements at each elicitation site using modified spring-loaded skin callipers
190 (Harpenden Skin-fold Calliper, British Indicators, England), as described (17). Skin-fold
191 thickness was recorded to the nearest 0.1 mm by the same investigator by placing the jaws of
192 the calliper at the outer diameter of the response site and measuring skin thickness only (no
193 subcutaneous fat).

194

Dermal thickness was determined at each patch site using a high-frequency ultrasound scanner (Episcan, Longport Inc., Reading, UK). The ultrasound probe was placed over the centre of each patch site together with ultrasound gel. The mean of three measurements was taken from each 12 mm scan image by an independent investigator, who was blinded to the trial assignment. Due to a delay in the availability of this equipment, dermal thickness was assessed in a subpopulation of 50 subjects who completed the DPCP patch test (CON = 13, 30MI = 14, 30HI = 12, 120MI = 11) and all subjects who completed the CO patch test.

Skin erythema is an objective measure of skin redness, which is considered the key measure of irritant responses (29). This was determined from triplicate measurement at each patch site using an erythema meter (ColorMeter DSM11, Cortex Technology, Hadsund, Denmark) as previously described (17).

Mean background values were determined from triplicate measurements at the vehicle only patch site for thickness and redness. In order to determine the increase in thickness and redness in response to DPCP or CO, the value from the vehicle-only site was subtracted from each patch site value. The values for increase in skin-fold thickness, dermal thickness and erythema over all the doses were summed, which gave an approximation of the area under the dose-response curve, representative of the overall reactivity of each subject to DPCP or CO, respectively.

Statistical analysis. Data in the results are presented as mean ± SD, unless otherwise stated and statistical significance was accepted at $P < 0.05$. Data were checked for normality and sphericity. Greenhouse-Geisser adjustments to the degrees of freedom were applied where necessary (skin-fold dose-series response to DPCP, epinephrine, norepinephrine and cortisol).

All statistical analysis was conducted using SPSS software. The mean difference with 95 % confidence intervals is presented for the main outcome measures.

Sample size was estimated using data from a previous study examining the effect of prior exercise stress on CHS responses to DPCP (17). The alpha (Type I error rate) was set at 0.05, and power at 0.95 (1 - Type II error rate) (G*Power software, version 3.1.2). For the CO element, a minimum important difference using biological variation data of the summed CO erythema response was used to estimate an effect size (0.91). A one-way ANOVA was used to assess differences between the groups in physical characteristics. The effect of exercise intensity and duration was analyzed using a one-way ANOVA to determine differences in the summed increase in responses to DPCP between the CON, 30MI, 30HI and 120MI trials. A two-way, mixed model ANOVA (DPCP data) or a repeated measures ANOVA (CO data) was used to analyze the skin-fold and dermal thickness responses across the full dose-series challenge (trial × dose). A two-way mixed model ANOVA (trial × time) was used to compare the circulating stress hormone data. Significant differences were identified using *post hoc* Tukeys HSD or Bonferroni corrected t-tests, where appropriate. To further investigate the differences between CON and 120MI, independent t-tests (DPCP data) or paired t-tests (CO data) were used to assess summed increases. Logarithmic transformation was performed on the DPCP data to allow for the calculation of the x-intercept when $y = 0$, utilizing linear regression on the linear portion of the dose response curve. A threshold dose for a response to DPCP was then calculated by back transformation (anti-log). Simple linear regression and a calculation of the standard error of the estimate (SEE) were performed to assess the validity of skin-fold measurement, using skin-fold callipers, as a practical method to determine dermal thickening compared with the objective criterion, high-frequency ultrasound. This was performed on the

sum of the 5 elicitation sites for a sub-population with complete data sets at the 48 h time point (n=50).

RESULTS

The effect of exercise intensity and duration on induction of DPCP immune memory.

Assessment of CHS responses. The skin-fold response, summed from five challenge doses, was significantly different between groups ($F(3,60) = 3.6$, $P < 0.05$). Tukeys post hoc analysis revealed that skin-fold thickness was reduced 67% by 120MI compared with CON ($P < 0.05$; Fig. 2a). The mean difference between 120MI and CON was 3.17 mm (95% confidence intervals 0.31 to 6.03 mm). There was no significant difference between the short duration 30MI or 30HI exercise groups compared with CON. The full, dose-series response to DPCP for each group was also determined for the increase in skin-fold thickness (Fig. 2b). The skin-fold thickness responses from the five individual doses revealed a significant trial × dose interaction ($F(7.3,145.1) = 3.0$, $P < 0.01$). Post hoc analysis revealed that skin-fold thickness was significantly lower in 120MI compared with CON at the 1.98 $\mu g \cdot cm^{-2}$ dose ($P < 0.05$), 5.08 $\mu g \cdot cm^{-2}$ and 8.12 doses ($P < 0.01$) and approached significance at the 3.17 $\mu g \cdot cm^{-2}$ dose ($P = 0.058$). To further investigate the differences between CON and 120MI, the threshold dose for a positive response to DPCP was calculated using the linear part of the dose response curve, as 0.48 and 2.09 $\mu g \cdot cm^{-2}$ for the CON and 120MI groups, respectively. This suggests that to elicit a positive response, 120MI required a 4.4 times greater DPCP dose in comparison with CON. Skin-fold thickness assessed using skin-fold callipers was strongly related with high-frequency ultrasound readings of dermal thickness ($r = 0.93$, $r^2 = 0.86$, SEE = 1.3 mm; $P < 0.01$).

*** Fig. 2 near here***

Circulating stress hormones. At baseline, pre-exercise, there were no significant differences between groups for circulating epinephrine, norepinephrine or cortisol concentration. A significant trial × time interaction was observed for circulating epinephrine ($F(4.6,88.5) = 7.0$,

$P < 0.01$; Fig. 3a), norepinephrine ($F(3.4,67.1) = 24.0$, $P < 0.01$; Fig. 3b) and cortisol concentration ($F(4.6,90.6) = 7.0$, $P < 0.01$; Fig. 3c). The raised circulating epinephrine and norepinephrine concentration observed immediately post on both 120MI and 30HI ($P < 0.01$) had returned to pre-exercise levels by 1 h post exercise. Circulating epinephrine concentration was greater at post on 120MI compared with CON ($P < 0.01$) and circulating norepinephrine concentration was greater at post on 30HI compared with CON ($P < 0.01$). Circulating cortisol concentration was greater at post and 1 h post on 120MI and at post on 30HI compared with CON ($P < 0.01$). The typical diurnal response in circulating cortisol concentration is shown, whereby levels were lower at post (1300) and 1 h post (1400) compared with pre-exercise (1100) on both 30MI and CON ($P < 0.01$).

*** Fig. 3 near here ***

The effect of prolonged, moderate intensity exercise on the induction of DPCP immune memory and cutaneous responses to the irritant, croton oil.

The aim here was to examine whether the inhibitory effect of 120MI on CHS is due to local effects on cutaneous inflammatory processes mediated principally via innate immune mechanisms. To this end, Fig. 4 shows the summed responses to all challenge doses for induction of DPCP immune memory (5 doses) and irritant responses to CO (4 doses). Results are presented as dermal thickness, considered a key measure of CHS responses (17), and erythema, considered a key measure of irritant responses (29). Here we show that 120MI significantly decreased DPCP responses measured as dermal thickness ($t(22) = 3.5$, $P < 0.01$; Fig. 4b) and erythema ($t(30) = 2.1$, $P < 0.05$; Fig. 4a). The mean difference for dermal thickness was 3.17 mm (95% confidence intervals 1.27 to 5.07) and for erythema was 18.61 AU (95% confidence intervals 0.41 to 36.82). No effect of 120MI-CO on irritant responses

measured as erythema ($t(10) = 0.2$, $P = 0.826$; Fig. 4c) or dermal thickness ($t(10) = 1.2$, $P = 0.253$; Fig. 4d) points to an inhibitory effect of 120MI on cell-mediated processes rather than local inflammatory processes in the decrease in CHS. It is noteworthy that the erythematous response to the top challenge dose of CO was comparable to the erythematous response to the top dose of DPCP (mean ± SD: 11.75 ± 5.28 AU and 11.25 ± 4.84 AU, respectively). As would be expected, dermal thickening response to the dose-series of the irritant, CO was small compared with DPCP (Fig. 4 d). For visual comparison, the increase in erythema responses to the full, dose-series of CO is also presented (Fig. 5). There was no significant trial × dose interaction observed between 120MI-CO and CON-CO for erythema responses ($F(3,30) = 1.4$, $P = 0.267$).

***Fig. 4 near here ***

***Fig. 5 near here ***

DISCUSSION

The advantages of, and the need for further research utilizing, *in vivo* immune measures in humans have recently been highlighted (1, 39). The primary aim of this work was to determine the unknown effects of the intensity and duration of continuous exercise stress on the induction of *in vivo* immunity in humans. In line with our hypothesis, prolonged, moderate intensity exercise (120MI) decreased the induction of *in vivo* immunity; however, short lasting moderate intensity (30MI) or high intensity (30HI) exercise did not influence this response despite elevated circulating catecholamines on 30HI and greater circulating cortisol on 30HI compared with CON. We then demonstrated that prolonged exercise had no effect on cutaneous responses to the irritant, CO. These findings support the notion that the observed decrease in *in vivo* immune induction to DPCP represents an effect on T-cell mediated immune responses rather than exercise-effects on local expression of inflammatory effector processes.

This is the first study to compare the effects of intensity and duration of continuous exercise stress on *in vivo* immunity assessed by use of an experimental CHS model in humans. In keeping with our previous findings, we observed that 120MI had a significant inhibitory effect on the induction of new immunity via the skin (17). Our finding that 30MI had no effect on *in vivo* immune induction is at odds with one hypothesis underpinning the J-shaped model (25), whereby a moderate dose of exercise is proposed to be immune-enhancing, but in accordance with recent research showing no effect of a moderate dose of exercise on the response to vaccination in young, healthy adults (20). While other studies have shown that a moderate dose of exercise can enhance antibody responses to vaccination, thereby supporting one hypothesis underpinning the J-shaped model, this typically occurs in individuals with sub-optimal immune status or when a half dose of vaccine is administered (14, 30). We also acknowledge that

exercise might differentially affect CHS, a cutaneous T-cell mediated response, and the antibody response to vaccination, a systemic B-cell mediated response.

We hypothesized that 30HI would decrease *in vivo* immune induction to DPCP, based upon evidence from *in vitro* work showing that short lasting high intensity exercise decreases indicators of both lymphocyte and neutrophil function (26, 32). However, our results do not support this despite elevated circulating catecholamines on 30HI and greater circulating cortisol on 30HI compared with CON. These findings provide little support for an involvement of circulating stress hormones in the mechanisms associated with altered *in vivo* immune responses to DPCP at the skin. For example, circulating norepinephrine was highest after 30HI when there was no immunosuppression suggesting that circulating norepinephrine has little immunosuppressive effect on the CHS system. Although circulating cortisol tended to be higher on 120MI compared with 30HI this did not reach statistical significance. In addition, circulating cortisol exceeded the purported binding capacity (~552 $nmol \cdot L^{-1}$)(22) at post-exercise before DPCP application in a similar proportion of subjects on 30HI (11 of 16) and 120MI (12 of 16) yet 30HI did not decrease immune induction by DPCP. There is clear evidence from murine models that high doses of these stress hormones can have significant immune-modulating effects. Intradermal injections of high dose corticosterone or catecholamines, both locally or distant from the sensitization site, inhibit the antigen-presenting capability of cutaneous DCs, reduce the number of T cells in draining lymph nodes and ultimately suppress DTH and CHS responses (11, 15, 33). Results from human studies are less consistent with some authors reporting a lack of association between stress hormones and *in vivo* immune responses (3, 13, 28). One frequently proposed explanation is that human studies typically rely on individual snapshot assessments of circulating stress hormones, thus missing important information regarding the kinetics of these responses. In this regard, we

acknowledge a limitation of the current study is that we applied the DPCP sensitization patch 20 minutes after exercise at a time when circulating cortisol likely reached a peak but circulating catecholamines would likely have returned to pre-exercise levels. At the outset, we considered the strengths and weaknesses of DPCP sensitization at the cessation of exercise to coincide with the peak in circulating catecholamines. After careful consideration, we chose to delay sensitization until 20 minutes after exercise to avoid possible confounding due to raised skin blood flow and sweating. One might also argue that another limitation is that we only took blood samples to characterize circulating cortisol at immediately post and 1 h post exercise yet the DPCP sensitizing patch remained in place for 48 h. Work in young adults showed the inhibitory effect of stress on the development of immune memory is particularly evident when stress is experienced at, or close to, the time of sensitization: this supports our choice of sample timing to characterize the circulating cortisol response in close proximity to the exercise stress (35).

The findings from the current study show that 120MI had no impact on cutaneous inflammatory responses to CO. This suggests that the inhibitory effect of 120MI on CHS induction with DPCP is likely associated with cell-mediated events rather than exercise effects on local inflammatory processes. Further research is required to better understand the mechanisms associated with the inhibitory effect of 120MI on *in vivo* responses to DPCP. Research should target the interactions between DC's and T cells in terms of antigen processing and presentation and activation of T cells and the subsequent balance between effector and regulatory T cells considered central to the successful induction of CHS (38). Also, the duration of the inhibitory effect of prolonged, heavy exercise on CHS induction in humans remains unknown and could be determined in a study that manipulates the timing of DPCP sensitization after 120MI. Given the reported sex differences in immune responses to

exercise (16), we recognize the limitation of using only males in this study and encourage the investigation of *in vivo* immune responses to exercise using this CHS model in females.

Experimental-CHS provides an attractive measure of *in vivo* immunity, not only because the skin is immediately accessible but because it overcomes many of the limitations of commonly used *in vitro* measures which are lacking in terms of clinical significance and practicality. We recognize that there are limitations with using DPCP in the CHS model described. Given that DPCP is benign, determining the clinical significance of the response, with specific regard to infection (skin and other) is an important avenue for future research. Preferably, the strength of the cutaneous recall response to DPCP could be generalized beyond skin immunity to indicate the immune system's general ability to respond to an infectious challenge. The available evidence in this regard is supportive as cutaneous immune measures are impaired in individuals with acute infectious illness (5), diabetes and psoriasis (4), and predict mortality in critically ill HIV-infected patients (12). An alternative viewpoint is that the benign characteristic of DPCP actually overcomes the ethical constraints associated with using live pathogens, such as rhinovirus to assess *in vivo* immunity. We also recognize the limitation that experimental-CHS requires purposefully inducing CHS; nevertheless, the selected doses we use are low and the mild elicitation responses are temporary.

Experimental-CHS with DPCP is practical, safe, and can be administered without the need for expensive equipment, invasive injections or blood sampling, making it a suitable immunological tool for both laboratory and field investigations. Moreover, the use of a novel antigen such as DPCP provides investigators with rigorous control over the timing and dose of sensitizing exposure, enabling the effects of various stressors on the primary immune response to be studied. The measurement of DTH responses to KLH is an alternative per-cutaneous *in*

420 *vivo* method, also reported to represent a primary immune response (36). However, since KLH
421 is derived from a shellfish this may explain why some individuals exhibit significant responses
422 to KLH prior to immunization (34). Experimental-CHS with DPCP is not restricted to
423 examining the effects of stress on the induction phase. Recently we have shown that this
424 approach can be used to assess the effect of exercise stress on the elicitation phase in subjects
425 who, following repeated monthly DPCP skin challenges, achieved a reproducible plateau in
426 responses (17). Furthermore, the standardized CHS model we describe overcomes some of the
427 limitations of vaccine models of *in vivo* immunity including variable immunogenicity (e.g.
428 hepatitis B (18)), annual changes in vaccine (e.g. influenza (7)) and difficulty when comparing
429 the circulating antibody results from different studies using in-house ELISAs. Nevertheless, a
430 standard protocol for measuring CHS elicitation responses in humans has yet to be established.
431 The use of erythema to quantify CHS elicitation has been questioned, particularly at sites of
432 stronger responses, where yellow vesicles can interfere with the erythema (redness) readings
433 (17, 24). Erythema is typically the preferred measure of irritant responses which, as we show
434 (Fig. 4 d), induce less edema than CHS responses (29). Notwithstanding the degree of
435 subjectivity, a particular strength of the current findings is that skin-fold thickness was strongly
436 related with dermal thickness measured by a high-frequency ultrasound scanner and read by a
437 blinded investigator (r = 0.93). Hence, we agree with the recommendation of others that, skin-
438 fold callipers present a simple and cost-effective measure of CHS edema (24).
439
440 In conclusion, using experimental CHS with DPCP, these results demonstrate that prolonged,
441 moderate intensity exercise, but not short-lasting high or short-lasting moderate intensity
442 exercise, decreases the induction of *in vivo* immunity in healthy humans. No effect of
443 prolonged, moderate intensity exercise on the skin's response to the irritant, CO points towards
444 a suppression of cell-mediated immunity in the observed decrease in CHS response. The

topical application of DPCP provides an attractive tool to assess the effect of exercise stress on *in vivo* immunity in humans.

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566 **Figure Legends**

567

568 FIGURE 1. Schematic for the effect of exercise intensity and duration on induction of DPCP

569 immune memory. Samples; venepuncture blood.

570

571 FIGURE 2. Effect of exercise stress prior to induction of contact hypersensitivity with DPCP

572 on responses to elicitation challenge 28 d later. Shown here as (a) summed increase in skin-fold

573 thickness (callipers: mean ± SD) and (b) responses to the full dose-series challenge with DPCP

574 on skin-fold thickness (callipers: mean ± SEM) # $P < 0.05$ and ## $P < 0.01$ *vs* CON. The

575 correlation was significant ($P < 0.01$).

576

577 FIGURE 3. Circulating epinephrine (a), norepinephrine (b) and cortisol (c) response to exercise

578 or seated rest. ↓= induction of contact sensitivity by DPCP application. ** $P < 0.01$ *vs* pre-

579 exercise; ## $P < 0.01$ *vs* CON. Data are mean ± SEM.

580

581 FIGURE 4. Effect of prolonged exercise stress (120MI) or seated rest (CON) prior to induction

582 of contact sensitivity with DPCP or irritant challenge with CO. Shown here are the summed

583 responses to: DPCP elicitation challenge 28 d later, measured as (a) erythema and (b) dermal

584 thickness (ultrasound); and CO challenge applied 20 minutes after exercise or equivalent seated

585 rest, measured as (c) erythema and (d) dermal thickness (ultrasound). # $P < 0.05$ and ## $P <$

586 0.01 *vs* CON. Data are mean ± SD.

587

588 FIGURE 5. Effect of prolonged exercise stress (120MI-CO) or seated rest (CON-CO) on

589 erythema responses to irritant challenge with CO. Shown here are the responses to the full

590 dose-series challenge with CO applied 20 minutes after exercise or equivalent seated rest. Data

591 are mean ± SEM.

592

593

TABLE 1. Subject information. Values are mean ± SD.

	CON	30MI	30HI	120MI
N	16	16	16	16
Age (years)	23 ± 4	20 ± 2	22 ± 4	22 ± 4
Height (cm)	180 ± 7	180 ± 5	179 ± 7	180 ± 7
Body mass (kg)	77.3 ± 11.3	74.5 ± 10.1	76.3 ± 12.8	78.8 ± 12.1
Body fat (%)	15.2 ± 3.7	15.1 ± 4.5	15.0 ± 4.7	15.9 ± 4.3
VO_{2peak} ($ml \cdot kg^{-1} \cdot min^{-1}$)	57 ± 7	58 ± 5	58 ± 6	56 ± 5
GET ($L \cdot min^{-1}$)	3.04 ± 0.31	3.09 ± 0.59	3.08 ± 0.60	3.11 ± 0.51
Weekly exercise (h)	6 ± 4	6 ± 2	5 ± 2	6 ± 3

GET, gas exchange threshold

Figure 1
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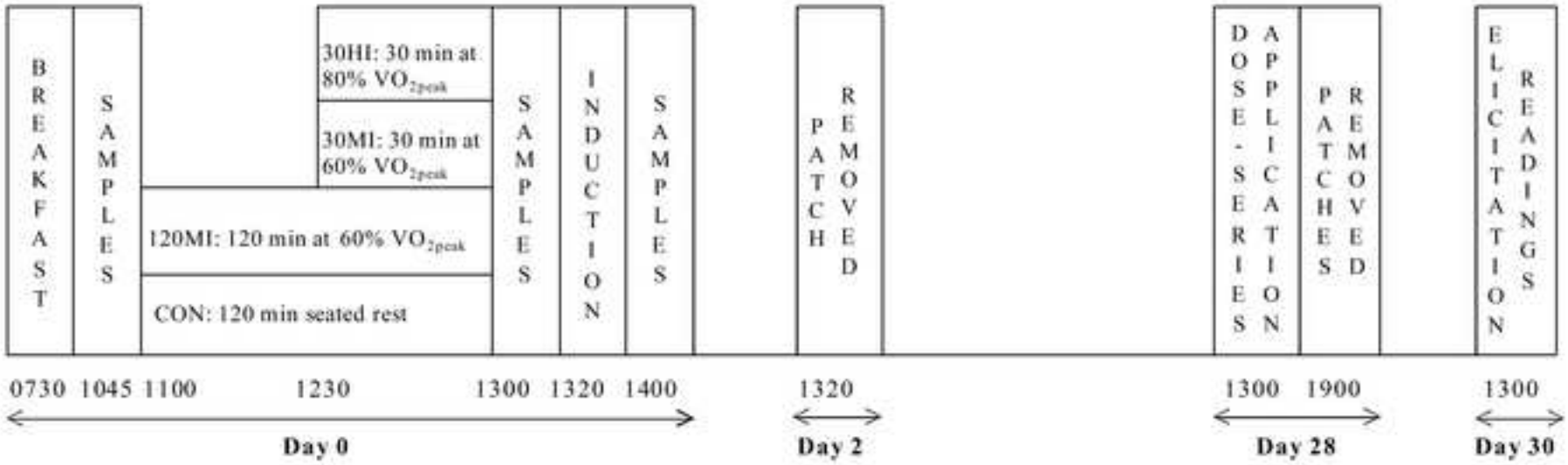

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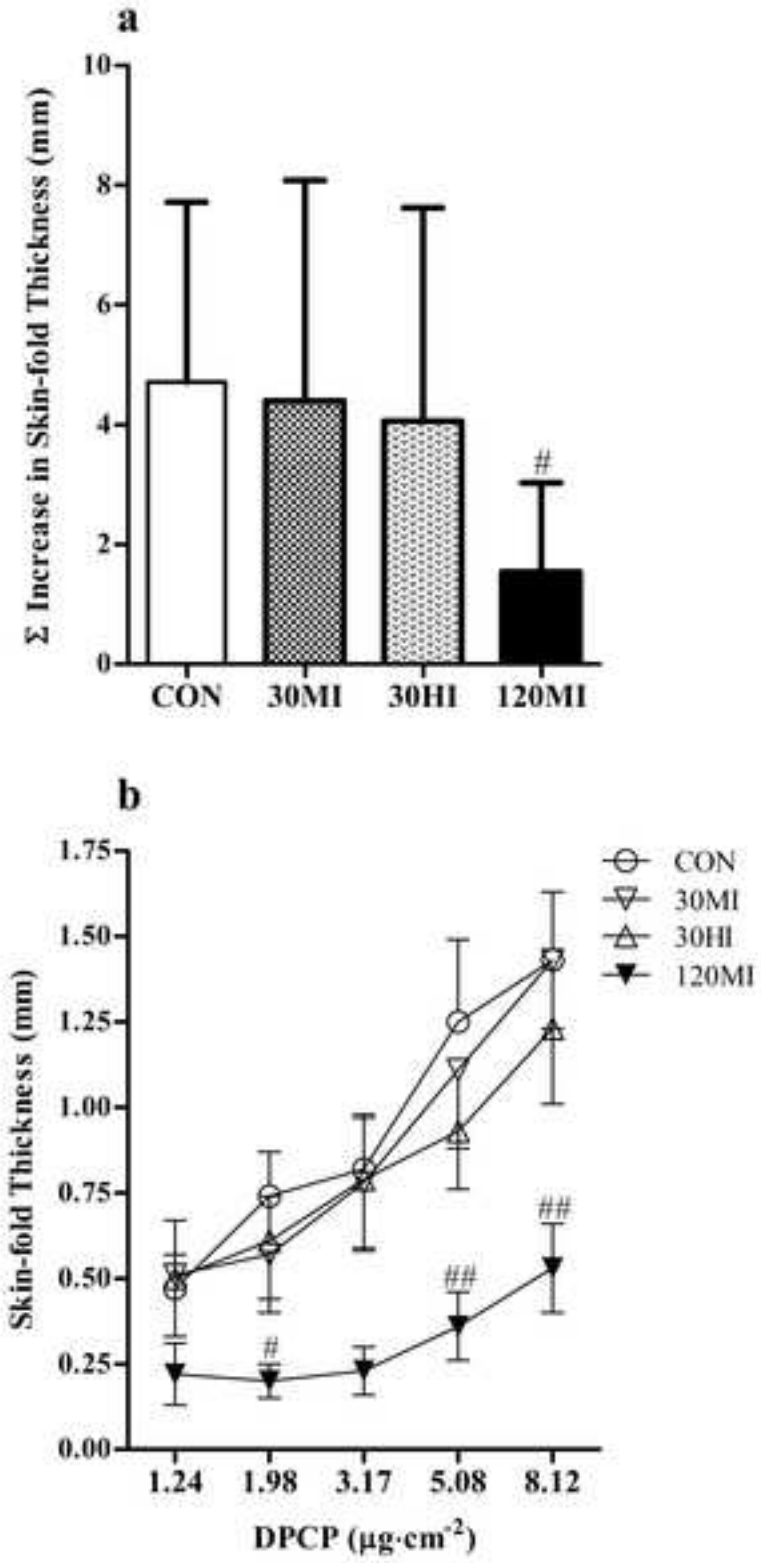

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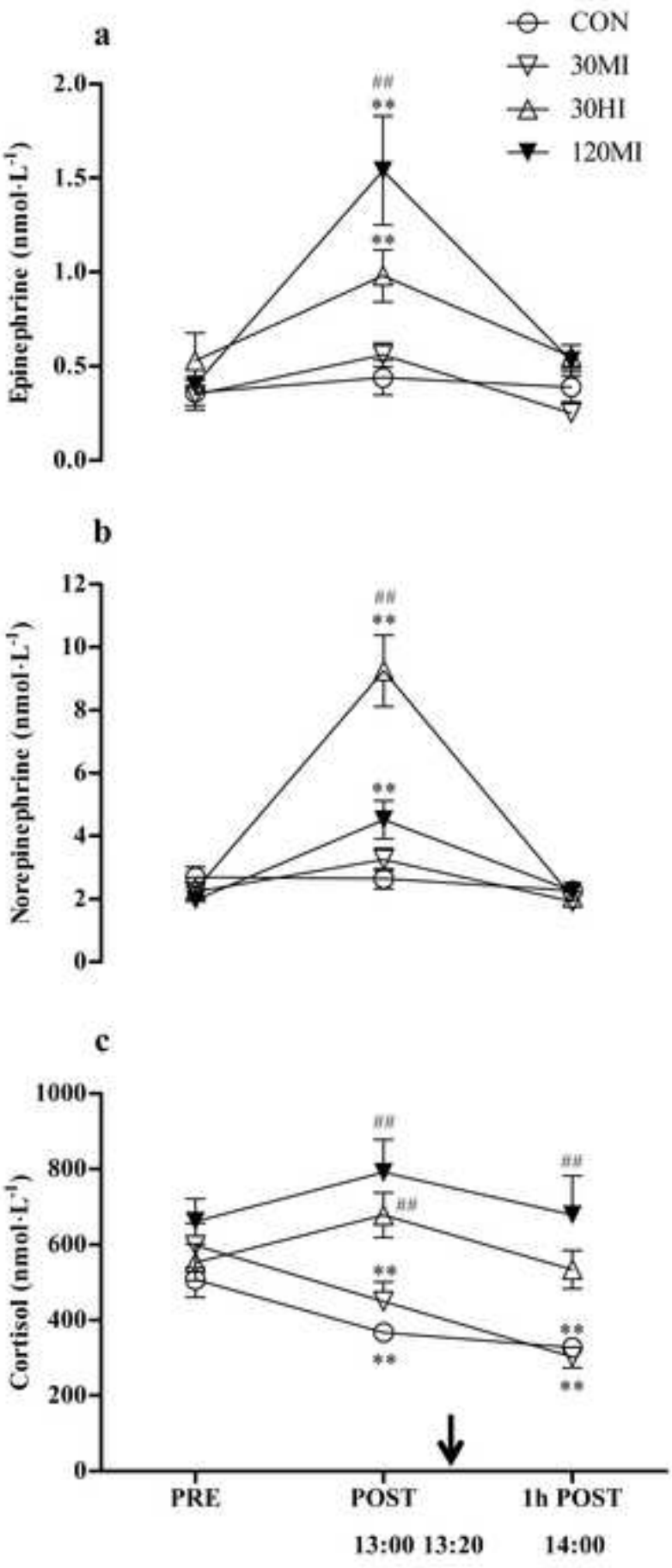

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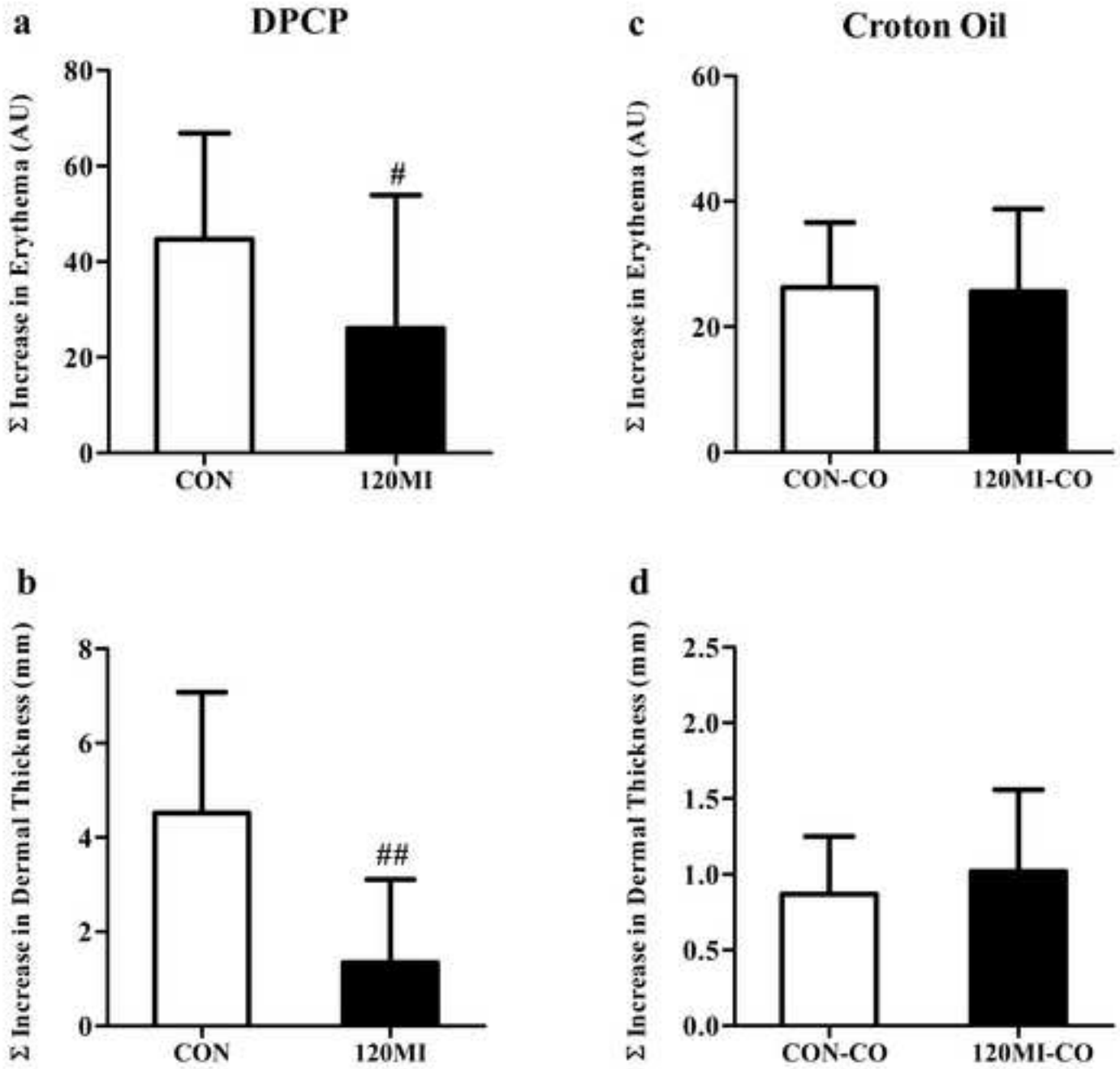

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